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INHERITANCE IN NICOTIANA. I. STUDY OF THE GLAUCOUS AND THE YELLOW CHARACTERS IN *N. TABACUM* L.

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INTRODUCTION

Several years ago the writer became interested in some morphological characters in *Nicotiana tabacum* L. while making preliminary studies with relative resistance of a number of tobacco varieties to tropical diseases. This study is therefore an outgrowth of another investigation. Materials have been accumulated steadily in our collection and although our interest is primarily of a phytopathological nature it has been and will be our purpose to render a genetical interpretation of such morphological characters as come to our attention and which appear not to have been described or analyzed genetically up to this time. We believe that the phytopathologist will make more rapid progress in studies on immunity which may lead to the development of disease-resistant strains of our crop plants when he knows more about the general genetical behavior of his plant materials. This knowledge will eliminate to a considerable extent many of the retarding factors which enter into the picture of the control of plant diseases by the use of resistant varieties, and consequently will simplify the methods, facilitate the planning of the experiments, shorten the period of the investigations and assure more prompt results.

This short paper proposes to constitute the first of what may become a series of studies on the genetics of Nicotiana. These studies were begun at the Insular Agricultural Experiment Station of Puerto Rico in 1928 and continued in Cornell University in 1930–32 and in the University of Wisconsin in 1932–33.

MATERIALS AND METHODS

The *glaucous* character reported herein occurs on a Puerto Rican

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commercial tobacco which has been known locally under the name of "Ceniza", meaning *ash*. The name refers to the peculiar appearance of the leaves which suggests a bluish or grayish green color. This character can be detected in the seed-bed at an early age but may be difficult to separate when seedlings are very crowded. For this reason, young seedlings should be transplanted to a second plant bed where more space will insure favorable conditions for development thereby minimizing the chances of failure in detecting the *glaucous* plants. Plants of this latter strain grow slower than ordinary tobaccos, the leaves are thick and the veins branch out from the midrib at an acute angle. The *glaucous* character appears not to have been described from any other country.

The *yellow* character as used here applies to a plant color which might more properly be designated as yellowish-green. This color is peculiar to normal plants at all stages of growth and is easily recognized in very young seedlings. The strain used in our studies is a pure line of a wrapper tobacco planted by a local company prior to the year 1927 in the tobacco districts at the higher altitudes of the island and which was believed to have descended from a single yellow plant discovered by a laborer in the back yard of his house. It is undoubtedly a mutation from a Puerto Rican variety. It was given the name of "Consolation" because it was discovered at a time when light wrappers were much in fashion among smokers and the industry here would have suffered tremendously had it not been for this timely discovery. But the advantage which the new tobacco brought to the growers was to dwindle away years later when the Consolation wrapper fell into disrepute. This yellow tobacco should not be confused with the *White Burley* tobacco studied by Henika (1) from which it differs in morphological expression and in being due to a single factor whereas the latter represents a two-factor difference. It seems also to be distinct from a tobacco variety which showed a golden color of leaves just before maturity and which was studied by Kajanus (2), who found an approximation to a genetical ratio of 15 : 1 for color.

The two characters reported herein were studied in the same crosses, thereby expediting the handling of a larger number of individuals for each character. The normal yellow tobacco is *non-glaucous* while the "Ceniza" or *glaucous*, being green, serves as a contrast to *yellow*. Other crosses which could not be carried beyond the second generation were also made with other *non-glaucous* or *non-yellow* pure lines.

In making counts for the yellow character it was only necessary to sow seed rather thinly in flats, then pricking out the seedlings with forceps when ten to fifteen days old. This method made possible the study of large numbers of seedlings. Another method employed was to transplant large plants from a seed-bed prepared in the usual way and making observations and counts in the field. It was soon found this method was unnecessary. In order to ascertain whether plants classified in the seed-bed as yellow or green were correctly determined, plantings of small seedlings grouped under the two classes were made in the greenhouse in one-and-a half-inch pots. Final counts made at the age of twenty-eight days, showed that the classification had been made accurately.

The separation of the *glaucous* or ashy seedlings was extraordinarily difficult in the seed-bed. It was found, however, that they could be detected in about a month after transplanting into small pots in the greenhouse, or into flats or beds when set at a longitudinal and transversal distance of two and one-half inches. They were transplanted into these when about two weeks old and counts could be made ten to fifteen days later. In doubtful cases the seedlings were further transplanted and kept for an additional period of two weeks when final counts were made on such individuals.

The same procedure was followed for all crosses and selfings.

In the determination of *ashy* or *glaucous* segregates the young seedlings were transplanted into flats of the usual size, transplanting thirty-five plants to each flat. The number of observations made in each progeny varied, depending upon the greenhouse and other facilities available at the time the different progenies were studied. In general, the population was fairly large.

In the season of 1928–1929 the following crosses were made: *glaucous* or "Ceniza" (Ce) $\times$ *yellow* or "*Consolation*" (Kon); green Turkish Sansum (T) $\times$ Kon; Ce $\times$ T; all direct and reciprocal. The first generations of these crosses were all grown at the Insular Experiment Station of Puerto Rico in the year 1928–29 and all selfings and backcrosses were then performed. The second and backcross generations were grown either at the Insular Experiment Station or in the greenhouse at Cornell University in the fall and winter of 1931; while further F_2 and third generations were grown in the spring and summer of 1932 at the latter institution. Third generation progenies were also studied at the Department of Horticulture, University of Wisconsin, in the summer of 1933.

The two characters, *yellow* and *glaucous,* are discussed separately. However, the data are from the same crosses.

In determining the significance of the genetical results given below, the probable error and Chi-square methods were employed. The probable error of numbers was determined in testing the significance of the 3:1 and 15:1 mendelian ratios. The expression Dev./P.E. was used as the measure of significance, taking Dev./P.E. = 3.2, as the lower level of significance, which represents odds of 31.36 to 1 against the occurrence of a deviation as great as or greater than the designated one. Values higher than 3.2 are regarded as giving odds higher than 31.36 that the deviations are not due to random sampling. In the application of the Chi-square method for goodness of fit, values of *P* were calculated by referring to Elderton's tables. It was considered that values of P equal to 0.05 or lower, indicated odds too high for such deviations to be due to random sampling.

RESULTS

The Yellow Character

The first generation of the crosses for the study of green and yellow was in all cases green, showing that the allelomorph *yellow* is a recessive character.

In the second generation of the direct and reciprocal crosses the seedlings were grouped into the two classes: green and yellow. From the data obtained in the F_2 of the cross *Ce* × *Kon* and *Kon* × *Ce* a hypothesis of a single factor relation between green and yellow was formulated. The figures are given in Table I, under *Kon* × *Ce* — A and *Ce* × *Kon* — A. In either case deviations can be attributed to random sampling.

Evidence from the Second generation.—In order to test the hypothesis, several F_2 progenies of the above cross were studied and in addition the cross *Kon* × *T* was made. Table I contains the distribution of the F_2 phenotypes in six progenies of the cross *Kon* × *Ce;* two progenies of the reciprocal cross and the direct and reciprocal cross *Kon* × *T*.

An examination of the figures shows a fairly close fit of the results to a 3:1 ratio of green to yellow seedlings. In only one progeny *Kon* × *Ce* — D is the deviation high enough to bring the results near the border line of significance. From the above F_2 results it seems evident that the single factor difference between green and yellow is appropriate in this case.

Evidence from the backcross.—Several of the F_1 plants which were selfed for F_2 studies were also backcrossed to the *yellow* parent. The F_1's of the cross *Kon* × *T* were backcrossed to no. 45, a *yellow* segregate from the cross *Ce* × *Kon* — A, which was also recessive for the "Ceniza" character. The latter are included although the crosses had been mainly intended for the study of the *Ce* character. These backcross progenies are given in Table II. Progeny ((*Kon* × *Ce*) × *Kon*)— B shows a deviation in the distribution of green and normal seedlings which appears too high to be attributed to random sampling, the D/P.E. being 3.62. The remaining eight backcross progenies show a fairly close agreement to the expected 1:1 ratio for single-factor differences. It is, therefore, safe to conclude that on the basis of backcross results a single-factor difference exists between green and *yellow*.

Evidence from the F_3 generation.—Further evidence in support of the single-factor pair difference for green and yellow is offered by the F_3 generation. In order to secure that evidence we studied thirty-four F_3 progenies of the cross *Ce* × *Kon* and thirty-four similar progenies of the reciprocal *Kon* × *Ce*. (See Tables III and IV). There should have been justification to regard all *yellow* segregants of the F_2 as pure breeding for that character. However, in the case of the cross *Ce* × *Kon*, *yellow* segregants were carried through the third generation so that in Table III we will find ten progenies of such segregants. This was not done with the similar segregates in the cross *Kon* × *Ce*. According to expectation the F_3 progenies should show a distribution of homozygous green to heterozygous green in the ratio of 1:2. An examination of Table III will reveal that such expectation was very closely realized, the green F_2 plants when carried through the third generation exhibiting a distribution of 8 pure-breeding green to 16 heterozygous individuals. The data in Table IV show a similar agreement with expectation; of 34 F_3 progenies, 23 proved to be heterozygous and 11 homozygous for green; a close approximation to the 2:1 ratio.

The hypothesis of a single-factor difference is further strengthened by the individual behavior in the F_3 of the heterozygous F_2 plants. Of sixteen such progenies in Table III only in family 26 are the results of doubtful significance. There the Dev./P. E. is almost 3.3 and therefore the odds against such a deviation being caused by random sampling are rather high. Whether a 3:1 ratio prevails in that family is not established by those results. All the 23 progenies in Table IV show a fairly good agreement with the expected 3:1 ratio.

From the evidence offered from F_2, B.C. and F_3 generations, a single-factor pair difference is established for *yellow* plant color as contrasted to green. It is proposed that this factor pair be designated *Yy*, green plants to be represented by *YY* and yellow plants by *yy*.

The Ceniza or Glaucous Character

The plants of the first generation crosses were all normal green or *non-glaucous*, indicating dominance of this character over *glaucous* (*Ce*).

In the study of the data on the *Ce* character two phenotypes were easily distinguishable, namely, the *glaucous* and *non-glaucous* and therefore individuals were classified into those two groups. The study of the distribution of the population of the second generation of the direct and reciprocal cross *Ce* × *Kon* and one backcross suggested a ratio of fifteen normal green plants to one "Ceniza" or *glaucous*. Progeny *Ce* × *Kon* — A (Table V) gave 438 normal and 35 *Ce* plants or a ratio of 12.51 to 1. The significance of these results rests on the fact that on the basis of a 15 : 1 ratio, the Dev./P.E. is only 1.53. Progeny *Kon* × *Ce* — A (Table V) showed a distribution of 128 normal to 10 *Ce* plants. These numbers represent a ratio of 12.8 to 1. On the assumption of a 15 : 1 ratio the Dev./P.E. is very low, only 0.72. The backcross progeny (*Kon* × *Ce*) × *Ce* given in Table V is represented by 251 normal to 66 *Ce* individuals, a ratio of 3.8 to 1. On the basis of a 3 : 1 ratio of normal to *Ce* the Dev./P.E. value is 2.55 and therefore such a deviation may be attributed to random sampling. The assumption of a segregation in the ratio of 15 normal to 1 *Ce* in the F_2 seems to be supported by a backcross ratio of 3 normal to 1 *Ce*. Therefore, there are sufficient grounds to assume a *two* factor pair difference between normal and *Ce*, the *Ce* character being exhibited only as the double recessive; and dominance of one or the other, or of both factors, producing normal plants.

Evidence from the second generation.—As proof of the hypothesis of the 15 : 1 relationship several F_2 progenies other than those reported above, were studied. (See Table V). Of 3 progenies of *Ce* × *Kon* only in B do the results appear to invalidate the hypothesis, with a Dev./P.E. reaching 3.37, slightly above the lower level of significance. But with larger numbers in C and D the results were in fairly good agreement with expectations, the Dev./P.E. being as low as 0.33 and 0.45, respectively. Other F_2 progenies which support the assumed 15 : 1 ratio are *Kon* × *Ce* — B, *45* × *T*, and *T* × *45*, A and B, and

T × *Ce*. All these progenies show a good agreement with the expected ratio.

Evidence from the backcrosses.—Additional evidence in support of the expected backcross ratio of 3:1 is furnished by progenies (*45* × *T*) × *45* and (*T* × *45*) × *45* which exhibit a D/P.E. of 1.48 and 1.75, respectively, and by (*T* × *Ce*) × *Ce* with a D/P.E. = 1.80.

Evidence from the third generation.—If the hypothesis of duplicate genes as an explanation of the results obtained in the F_2 and B.C. generations of crosses involving the study of the *Ce* character holds, it would be expected that in the third generation some progenies should be normal, some should segregate in the ratio of 15:1 and some 3:1, normal to *Ce*; while one out of sixteen should be *glaucous*. That this expectation was realized is evidenced by the data on 20 progenies of the cross *Ce* × *Kon* (Table VI) and 28 progenies of the reciprocal (Table VII). An examination of Table VI reveals that out of 20 progenies, seven segregated in the ratio of 15 to 1, six in the ratio of 3:1, five were pure breeding normal and two were "Ceniza" or *glaucous*. In the segregating families the statistical analysis of the data proves that the results significantly support the assumption of either 15:1 or 3:1 ratios. A similar behavior is obtained in the F_3 of the reciprocal cross (Table VII). There, seven progenies segregated in a ratio of 15 to 1 and six in the ratio of 3:1 normal to *glaucous;* thirteen were pure breeding normal while two were "Ceniza" or *glaucous*. If all the F_3 progenies are considered together the distribution is as follows: 18 progenies pure breeding normal, 14 progenies segregating in the ratio of 15:1, 12 others in the ratio of 3:1, and four homozygous *glaucous*. If the composition of a normal plant be represented by Ce_1 Ce_1 Ce_2 Ce_2, the *glaucous* by ce_1 ce_1 ce_2 ce_2 and the hybrid in the cross by Ce_1 ce_1 Ce_2 ce_2, the following genotypes and ratios would be expected in F_3 on the basis of the 15:1 hypothesis: 1 Ce_1 Ce_1 Ce_2 Ce_2, 2 Ce_1 Ce_1 Ce_2 ce_2, 1 Ce_1 Ce_1 ce_2 ce_2, 2 Ce_1 ce_1 Ce_2 Ce_2, 4 Ce_1 ce_1 Ce_2 ce_2, 2 Ce_1 ce_1 ce_2 ce_2, 1 ce_1 ce_1 Ce_2 Ce_2, 2 ce_1 ce_1 Ce_2 ce_2, and 1 ce_1 ce_1 ce_2 ce_2. Of those with either one or the other factor, or both factors in the dominant condition, Ce_1 ce_1 Ce_2 ce_2 would be expected to segregate in the ratio of 15:1, Ce_1 ce_1 ce_2 ce_2 and ce_1 ce_1 Ce_2 ce_2 in the ratio of 3:1, ce_1 ce_1 ce_2 ce_2 would be *glaucous* and all the other genotypes would be pure breeding normal. There would then be expected four classes of genotypes in the following ratios: 7 pure breeding normal, 4

segregating 15:1, 4 segregating 3:1 and 1 pure recessive. When the Chi-square test for goodness of fit is applied to the expected results given above, a value of P = 0.78 is obtained.

Class	Expected Ratio	Frequencies		$\frac{O-C^2}{C}$	P
		Observed	Calculated		
Normal	7	18	21		
15:1 ratio	4	14	12		
3:1 ratio	4	12	12		
Pure Recessive	1	4	3		
Total	16	48	48	1.09523	0.78046

The deviations from the expected ratio of the distribution of the F_3 families are such as might be expected by chance alone eleven times in twenty trials and it may be concluded, therefore, that the results obtained support the suggested hypothesis of duplicate genes.

On the basis of F_2, B.C. and F_3 results it is concluded that the assumption of a 2-factor difference for the *Ce* character is justified. These factors are designated as Ce_1 and Ce_2 with their corresponding allelomorphs ce_1 and ce_2. The presence of either factor in the dominant condition produces normal plants while both factors must be recessive to produce the "Ceniza" character. The constitution of a "Ceniza" plant will be represented by $ce_1\ ce_1\ ce_2\ ce_2$.

Independent Inheritance

It was of interest to determine from the data whether there existed any linkage relations between the factor pair Yy and $Ce_1\ ce_1$ or $Ce_2\ ce_2$. The fact that *N. tabacum* has twenty-four pairs of chromosomes would make the detection of linkage between those factors of unique interest especially since the occurrence of the mutants has been reported from Puerto Rico simultaneously. From the analysis of the distribution of the phenotypes in three F_2 progenies, twelve F_3's and one backcross progeny it appears that no linkage exists between the factor pairs $Ce_1\ ce_1$ and $Ce_2\ ce_2$ and yellow (Yy). The reader is referred to Table VIII. The F_2 progenies *Ce* × *Kon* — C and — D and *Kon* × *Ce* —A, on the basis of independent inheritance of the characters should yield four classes in the following ratios: 45 normal green, 15 normal yellow, 3 *Ce* green and 1 *Ce* yellow. The Chi-square method of testing the goodness of fit showed that for *Ce* × *Kon* — *C*, P = 0.53; for *Ce* × *Kon* — D, P = 0.80 and *Kon* × *Ce* — A, P = 0.52. These values of P all show that the deviations from the expected are not significant.

In the F_3 of the direct and reciprocal crosses of *Ce* × *Kon*, progenies 6, 7, 52, 67, 68 and 76, segregating in the ratio of 3 normal to 1 *Ce* and 3 green to 1 yellow, should show a distribution of phenotypes as follows: nine normal green, 3 normal yellow, 3 *Ce* green and 1 *Ce* yellow. These expectations are realized in all these progenies (see Table VIII). In all these progenies the values of P are high, the lowest being 0.13 for family no. 68. This value, however, indicates that the deviations might be expected to be due to chance alone, once in eight times.

Progenies 30, 55, 62, 66, 71 and 85 which segregated in the ratio of 15 normal to 1 *Ce* and 3 green to 1 yellow, would be expected to show the phenotypes in the same ratios as the F_2 progenies, namely 45:15:3:1. Such expectation is fully accomplished in all the progenies except no. 55. An examination of the distribution of the phenotypes in that progeny, however, shows that the deficiencies may not be attributed to linkage.

The distribution of the genotypes in the F_3 generation is given in Table IX. The normal expectation of genotypes on the basis of a 15:1 ratio for the *Ce* character and 3:1 segregation for green and yellow is as follows. Green genotypes to give only, 7 normal; 4 segregating in the ratio of 15 normal to 1 *Ce*; 4 in the ratio of 3 normal to 1 *Ce;* and 1 pure breeding "Ceniza". Those green individuals of the composition *Yy* should show the following distribution: 14 normal, 8 segregating 15 normal to 1 *Ce,* 8 segregating into 3 normal and 1 *Ce,* and 2 pure breeding *Ce.* The yellow genotypes are not analyzed in the above table, but the expectation would be the same as for pure breeding greens.

A consideration of the results given in Table IX shows that the deviations from the expected ratio are such as can well be attributed to conditions of the experiment.

The results of F_2 and F_3 progenies given in support of the hypothesis of the independent inheritance of the Yy and Ce_1 ce_1 Ce_2 ce_2 are further complemented by the backcross results.

So, from the above results it may safely be concluded that no linkage exists between the factor for *yellow* plant color and those factors responsible for the "Ceniza" or *glaucous* character.

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SUMMARY

1. Two previously undescribed characters of *N. tabacum* are described and studied genetically. The *yellow* plant color appears to be distinct from similar deficiencies heretofore reported. It is not to be confused with the *Burley* character in White Burley tobacco.

2. Green color is dominant to *yellow* color.

3. Normal green is dominant to *glaucous* or "Ceniza".

4. Green and *yellow* are differentiated by a single factor pair which is designated *Yy*.

5. Normal green and *Ce* or *glaucous* plants are differentiated by two factor pairs; therefore segregation occurs in the proportion of 15 normal green to 1 *glaucous* (*Ce*). These are designated by Ce_1 ce_1 Ce_2 ce_2.

6. The factors for *glaucous* and for *yellow* are inherited independently of each other.

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APPENDIX

TABLE I

SEGREGATION OF THE GREEN (Y) AND *YELLOW* (y) CHARACTERS IN THE F_2 OF CROSSES BETWEEN VARIOUS PURE LINES OF TOBACCO

Progeny	Color	n	Frequencies		Dev.	P. E.	D/P. E
			Observed	Calculated (3:1)			
Kon x Ce—A.....	Green	461	359	345.75	13.25	6.27	2.11
	Yellow....		102	115.25			
Kon x Ce—B.....	Green.....	1995	1489	1496.25	7.25	13.08	0.55
	Yellow....		506	498.75			
Kon x Ce—C.....	Green.....	400	297	300.00	3.00	5.84	0.51
	Yellow....		103	100.00			
Kon x Ce—D.....	Green.....	912	656	684.00	28.00	8.82	3.17
	Yellow....		256	228.00			
Kon x Ce—E.....	Green.....	824	638	618.00	20.00	8.38	2.39
	Yellow. ..		186	206.00			
Kon x Ce—F.....	Green.....	713	536	534.75	1.25	7.80	0.16
	Yellow....		177	178.25			
Ce x Kon—A.....	Green.....	1150	856	862.50	6.50	9.90	0.66
	Yellow....		294	287.50			
Ce x Kon—B.....	Green.....	358	279	268.50	10.50	5.53	1.89
	Yellow....		79	89.50			
Kon x T—A.....	Green.....	4642	3522	3481.50	40.50	19.90	2.04
	Yellow....		1120	1160.50			
T x Kon—B....	Green.....	5156	3844	3867.00	23.00	20.97	1.10
	Yellow....		1312	1289.00			

TABLE II

SEGREGATION OF THE GREEN (Y) AND *YELLOW* (y) CHARACTERS IN THE BACK-CROSS GENERATION OF CROSSES BETWEEN VARIOUS PURE LINES OF TOBACCO

Progeny	Color	n	Frequencies		Dev.	P. E.	D/P. E.
			Observed	Calculated (1:1)			
(Kon x Ce) x Kon—A.	Green.....	1797	869	898.50	29.50	14.29	2.06
	Yellow....		928	898.50			
(Kon x Ce) x Kon—B.	Green.....	730	398	365.00	33.00	9.11	3.62
	Yellow....		332	365.00			
(Kon x Ce) x Kon—C.	Green.....	974	490	487.00	3.00	10.53	0.28
	Yellow....		484	487.00			
(Kon x Ce) x Kon—D.	Green.....	141	64	70.50	6.50	4.00	1.62
	Yellow....		77	70.50			
(Kon x Ce) x Kon—E.	Green.....	367	184	183.50	0.50	6.46	0.08
	Yellow....		183	183.50			
(Ce x Kon) x Kon—A.	Green.....	154	80	77.00	3.00	4.19	0.72
	Yellow....		74	77.00			
(Ce x Kon) x Kon—B.	Green.....	362	191	181.00	10.00	6.42	1.56
	Yellow....		171	181.00			
(Kon x T) x 43.....	Green.....	911	460	455.50	4.50	10.18	0.44
	Yellow....		451	455.50			
T x Kon) x 43.....	Green.....	1187	589	593.50	4.50	11.62	0.36
	Yellow....		598	593.50			

TABLE III

RESULTS OF THE F_3 GENERATION OF THE CROSS *Ce* × *Kon* IN THE STUDY OF THE *YELLOW* CHARACTER

Progeny	Color	n	Frequencies		Dev.	P. E.	D/P. E.
			Observed	Calculated (3:1)			
2	Green	996	776	747.00	29.00	9.22	3.15
	Yellow		220	249.00			
4	Green	920	708	690.00	18.00	8.86	2.03
	Yellow		212	230.00			
6	Green	1135	876	851.25	24.75	9.84	2.52
	Yellow		259	283.75			
7	Green	470	359	352.50	6.50	6.33	1.03
	Yellow		111	117.50			
8	Green	483	373	362 25	10.75	6.42	1.67
	Yellow		110	120.75			
10	Green	144	115	108.00	7.00	3.50	2 00
	Yellow		29	36.00			
13	Green	517	393	387.75	5.25	6.64	0.79
	Yellow		124	129.25			
21	Green	1353	1033	1014.75	18.25	10.71	1.70
	Yellow		320	338.25			
25	Green	158	120	118.50	1.50	3.67	0.41
	Yellow		38	39.50			
26	Green	689	542	516.75	25.25	7.67	3.29
	Yellow		147	172.25			
31	Green	459	363	344.25	18.75	6.26	2.99
	Yellow		96	114.75			
32	Green	333	241	249.75	8.75	5.33	1.64
	Yellow		92	83.25			
33	Green	1250	966	937.50	28.50	10.30	2.77
	Yellow		284	312.50			
34	Green	202	150	151.50	1.50	4.15	0.36
	Yellow		52	50.50			
44	Green	406	315	304.50	10.50	5.88	1.79
	Yellow		91	101.50			
46	Green	751	560	563.25	3.25	8.00	0.41
	Yellow		191	187.75			

Families breeding true to green: 1, 5, 9, 14, 20, 23, 45 and 47.

Families breeding true to yellow: 15, 16, 17 18, 19, 39, 40, 41, 42 and 43

TABLE IV

BEHAVIOR OF THE F_1 GENERATION OF THE CROSS *Kon* × *Ce* IN THE STUDY OF THE *YELLOW* CHARACTER

Progeny	Color	n	Frequencies		Dev.	P. E.	D/P. E.
			Observed	Calculated (3:1)			
52	Green	585	445	438.75	6.25	7.06	0.89
	Yellow		140	146.25			
54	Green	376	291	282.00	9.00	5.66	1.59
	Yellow		85	94.00			
55	Green	1016	772	762.00	10.00	9.31	1.07
	Yellow		244	254.00			
56	Green	492	373	369.00	4.00	6.48	0.62
	Yellow		119	123.00			
61	Green	761	566	570.75	4 75	8.06	0.59
	Yellow		195	190.25			
62	Green	759	570	569.25	0.75	8.05	0 09
	Yellow		189	189.75			
63	Green	439	335	329.25	5.75	6.12	0.94
	Yellow		104	109.75			
66	Green	452	339	339.00	0	6.21	0
	Yellow		113	113.00			
67	Green	405	302	303.75	1.75	5.88	0 30
	Yellow		103	101.25			
68	Green	416	318	312.00	6.00	5.96	1.01
	Yellow		98	104.00			
69	Green	497	375	372.75	2.75	6.51	0.42
	Yellow		122	124.25			
70	Green	435	332	326.25	5.75	6.09	0.94
	Yellow		103	108.75			
71	Green	325	253	243.75	9.25	5.27	1.76
	Yellow		72	81.25			
72	Green	213	153	159.75	6.75	4.26	1.58
	Yellow		60	53.25			
76	Green	208	154	156.00	2.00	4.21	0.48
	Yellow		54	52.00			
77	Green	208	155	156.00	1.00	4.21	0.24
	Yellow		53	52 00			
79	Green	260	202	195 00	7.00	4.71	1.49
	Yellow		58	65.00			
85	Green	360	270	270 00	0	5.54	0
	Yellow		90	90 00			
87	Green	359	284	269.25	14.75	5.53	2.67
	Yellow		75	89.75			
88	Green	248	191	186 00	5.00	4.60	1.09
	Yellow		57	62 00			
89	Green	266	210	199.50	10.50	4.76	2.20
	Yellow		56	66.50			
90	Green	549	428	411 75	16.25	6.84	2.38
	Yellow		121	137 25			
91	Green	196	149	147 0(	2.0(	4.0.	0.49
	Yellow		47	49.0(			

Families breeding true to green: 53, 57, 58, 59, 60, 64, 65, 74, 78, 81 and 83.

TABLE V

SEGREGATION OF THE CENIZA (ce) OR *GLAUCOUS* CHARACTER AND THE NORMAL CHARACTER (Ce) IN THE SECOND AND BACKCROSS GENERATIONS OF CROSSES

Progeny	Color	n	Frequencies			Dev.	P. E.	D/P. E.
			Observed	Calculated				
				n	ratio			
Ce x Kon—A.....	Normal...	473	438	443.44	15:1	5.44	3.55	1.53
	Ceniza....		35	29.56				
Ce x Kon—B.....	Normal...	451	411	422.71	15:1	11.71	3.47	3.37
	Ceniza....		40	28.29				
Ce x Kon—C.....	Normal...	997	933	934.69	15:1	1.69	5.16	0.33
	Ceniza....		64	62.31				
Ce x Kon—D.....	Normal...	981	922	919.69	15:1	2.31	5.11	0.45
	Ceniza....		59	61.31				
Kon x Ce—A.....	Normal...	138	128	129.38	15:1	1.38	1.92	0.72
	Ceniza....		10	8.62				
Kon x Ce—B.....	Normal...	181	167	169.69	15:1	2.69	2.20	1.22
	Ceniza....		14	11.31				
45 x T.....	Normal...	85	81	79.69	15:1	1.31	1.51	0.87
	Ceniza....		4	5.31				
T x 45—A.....	Normal...	246	229	230.62	15:1	1.62	2.56	0.63
	Ceniza....		17	15.38				
T x 45—B.....	Normal..	210	199	196.87	15:1	2.12	2.37	0 90
	Ceniza....		11	13.12				
T x Ce.....	Normal...	292	266	273.75	15:1	7.75	2.69	2.88
	Ceniza....		26	18.25				
(T x Ce) x Ce.....	Normal...	379	274	284.25	3:1	10.25	5.69	1.80
	Ceniza....		105	94.75				
(Kon x Ce) x Ce..	Normal...	317	251	237.75	3:1	13.25	5.20	2.55
	Ceniza....		66	79.25				
(45 x T) x 45.....	Normal...	66	53	49.50	3:1	3.50	2.37	1.48
	Ceniza....		13	16.50				
(T x 45) x 45.....	Normal...	175	138	131.25	3:1	6.75	3.86	1.75
	Ceniza....		37	43.75				

TABLE VI

BEHAVIOR OF THE F_2 PROGENIES OF THE CROSS *Ce* × *Kon* IN THE STUDY OF THE *CENIZA* OR *GLAUCOUS* CHARACTER

Progeny	Color	n	Frequencies			Dev.	P. E.	D/P. E.
			Observed	Calculated				
				n	ratio			
17	Normal	137	133	128.440	15:1	4.560	1.91	2.39
	Ceniza		4	8.560				
18	Normal	34	31	31.880	15:1	0.880	0.95	0.93
	Ceniza		3	2.120				
19	Normal	55	51	51.560	15:1	0.560	1.21	0.46
	Ceniza		4	3.440				
23	Normal	130	122	121.875	15:1	0.125	1.86	0.07
	Ceniza		8	8.125				
30	Normal	120	114	112.500	15:1	1.500	1.79	0.84
	Ceniza		6	7.500				
40	Normal	128	122	120.000	15:1	2.000	1.85	1.08
	Ceniza		6	8.000				
42	Normal	128	123	120.000	15:1	3.000	1.85	1.62
	Ceniza		5	8.000				
5	Normal	246	181	184.500	3:1	3.500	4.58	0.76
	Ceniza		65	61.500				
6	Normal	175	138	131.250	3:1	6.750	3.86	1.75
	Ceniza		37	43.750				
7	Normal	153	111	114.750	3:1	3.750	3.61	1.04
	Ceniza		42	38.250				
16	Normal	153	141	143.440	3:1	2.440	3.61	0.68
	Ceniza		12	9.560				
25	Normal	120	94	90.000	3:1	4.000	3.20	1.25
	Ceniza		26	30.000				
32	Normal	120	96	90.000	3:1	6.000	3.20	1.87
	Ceniza		24	30.000				

Families breeding true to normal: 4, 9, 15, 26 and 33.

Families breeding true to "Ceniza" or *glaucous*: 39 and 43.

TABLE VII

BEHAVIOR OF THE F_3 PROGENIES OF THE CROSS *KON* x *CE* IN THE STUDY OF THE CENIZA OR *GLAUCOUS* CHARACTER

Progeny	Color	n	Frequencies			Dev.	P. E.	D/P. E.
			Observed	Calculated				
				n	ratio			
55	Normal	365	334	342.19	15:1	8.19	3.12	2.62
	Ceniza		31	22.81				
62	Normal	282	264	264.32	15:1	0.37	2.74	0.14
	Ceniza		18	17.62				
65	Normal	311	299	291.56	15:1	7.44	2.88	2.58
	Ceniza		12	19.44				
66	Normal	448	421	420.00	15:1	1.00	3.46	0.29
	Ceniza		27	28.00				
71	Normal	308	281	288.75	15:1	7.75	2.87	2.70
	Ceniza		27	19.25				
85	Normal	286	268	268.13	15:1	0.13	2.76	0.05
	Ceniza		18	17.87				
91	Normal	149	142	139.69	15:1	2.31	1.99	1.16
	Ceniza		7	9.31				
52	Normal	401	319	300.75	3:1	18.25	5.85	3.12
	Ceniza		82	100.25				
58	Normal	327	258	245.25	3:1	12.75	5.28	2.41
	Ceniza		69	81.75				
67	Normal	380	287	285.00	3:1	2.00	5.69	0.35
	Ceniza		93	95.00				
68	Normal	406	313	304.50	3:1	8.50	5.88	1.45
	Ceniza		93	101.50				
76	Normal	198	155	148.50	3:1	6.50	4.11	1.58
	Ceniza		43	49.50				
83	Normal	252	201	189 00	3:1	12.00	4.64	2.59
	Ceniza		51	63.00				

Families breeding true to normal: 53, 57, 60, 63, 64, 69, 70, 72, 77, 78, 79. 89 and 90.

Families breeding true to "Ceniza": 59 and 61.

TABLE VIII

SHOWING PHENOTYPIC DISTRIBUTION IN F_2, F_3 AND B. C. PROGENIES OF CROSSES IN THE STUDY OF THE *YELLOW* AND CENIZA CHARACTERS

Progeny	Class *	Frequencies			Chi Square	P
		Observed	Calculated			
			ratio	n		
Ce x Kon—C	NY	703	45	701.0160		
	Ny	230	15	233.6720		
	nY	43	3	46.7340		
	ny	21	1	15.5780		
	Total	997		997.0000	=2.24376	0.53
Ce x Kon—D	NY	693	45	689.7660		
	Ny	229	15	229.9220		
	nY	41	3	45.9840		
	ny	18	1	15.3280		
	Total	981		981.0000	=1.02484	0.80
Kon x Ce—A	NY	100	45	97.0310		
	Ny	28	15	32.3440		
	nY	9	3	6.4688		
	ny	1	1	2.1562		
	Total	138		138.0000	=2.28431	0.52
Ce x Kon—6	NY	94	9	98.4375		
	Ny	44	3	32.8125		
	nY	29	3	32.8125		
	ny	8	1	10.9375		
	Total	175		175.0000	=5.24636	0.16
Ce x Kon—7	NY	81	9	86.0625		
	Ny	30	3	28.6875		
	nY	35	3	28.6875		
	ny	7	1	9.5625		
	Total	153		153.0000	=2.43355	0.49
Ce x Kon—30	NY	49	45	42.1875		
	Ny	8	15	14.0625		
	nY	2	3	2.8125		
	ny	1	1	0.9375		
	Total	60		60.0000	=3.95250	0.27
Kon x Ce—55	NY	226	45	256.6410		
	Ny	119	15	85.5470		
	nY	12	3	17.1090		
	ny	8	1	5.7030		
	Total	365		365.0000	=19.19081	0.0003
Kon x Ce—62	NY	196	45	198.2810		
	Ny	68	15	66.0940		
	nY	12	3	13.2190		
	ny	6	1	4.4060		
	Total	282		282.0000	=1.26496	0.74
Kon x Ce—66	NY	322	45	315.0000		
	Ny	99	15	105.0000		
	nY	22	3	21.0000		
	ny	5	1	7.0000		
	Total	448		448.0000	=1.11746	0.77

*The class *NY* stands for normal green, *Ny* is normal yellow, *nY* is ce, or *glaucous* green, and *ny* represents ce yellow.

TABLE VIII—(Cont.)

SHOWING PHENOTYPIC DISTRIBUTION IN F_2, F_3 AND B. C. PROGENIES OF CROSSES IN THE STUDY OF THE *YELLOW* AND CENIZA CHARACTERS

Progeny	Class *	Frequencies			Chi Square	P
		Observed	Calculated			
			ratio	n		
Kon x Ce—71	NY	217	45	216.5620		
	Ny	64	15	72.1870		
	nY	22	3	14.4380		
	ny	5	1	4.8130		
	Total	308		308.0000	=4.89732	0.18
Kon x Ce—85	NY	186	45	201.0940		
	Ny	82	15	67.0310		
	nY	13	3	13.4060		
	ny	5	1	4.4690		
	Total	286		286.0000	=4.55113	0.21
Kon x Ce—52	NY	223	9	225.5620		
	Ny	96	3	75.1870		
	nY	59	3	75.1880		
	ny	23	1	25.0630		
	Total	401		401.0000	=9.44557	0.24
Kon x Ce—67	NY	207	9	213.7500		
	Ny	80	3	71.2500		
	nY	72	3	71.2500		
	ny	21	1	23.7500		
	Total	380		380.0000	=1.61403	0.66
Kon x Ce—68	NY	239	9	228.3750		
	Ny	74	3	76.1250		
	nY	79	3	76.1250		
	ny	14	1	25.3750		
	Total	406		406.0000	=5.76136	0.13
Kon x Ce—76	NY	123	9	111.3750		
	Ny	32	3	37.1250		
	nY	31	3	37.1250		
	ny	12	1	12.3750		
	Total	198		198.0000	=2.94275	0.40

TABLE IX

BEHAVIOR OF GENOTYPES IN RELATION TO THE *CE* CHARACTER OR ITS ALLELOMORPH IN F_2 FAMILIES WHICH ARE PURE BREEDING FOR GREEN OR SEGREGATING FOR GREEN AND YELLOW

Class		Calculated ratio	n		Chi Square	P
			Observed	Calculated		
Green	Normal...	7	6	5.250		
	15:1.....	4	2	3.000		
	3:1.....	4	3	3.000		
	ce.....	1	1	0.750		
	Total.....	16	12	12.000	=0.52380	Over 0.80
Segregating Green & Yellow	Normal...	14	11	11.375		
	15:1.....	8	6	6.500		
	3:1.....	8	8	6.500		
	ce.....	2	1	1.625		
	Total.....	32	26	26.000	=0.63734	Over 0.80

THE UTILIZATION OF WASTE MOLASSES IN THE PRODUCTION OF

I. ACETONE AND BUTANOL
II. NORMAL BUTYRIC ACID

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INTRODUCTION

The industrial world has felt the influence of fermentation processes, especially during the past thirty years; and is beginning to realize the tremendous significance of the application of industrial microbiology in the development of useful chemicals from cellulose, starch and sugars.

The microbiological flora of the tropics is rich in organisms capable of industrial utilization, and the industry of the sugar cane offers abundant and inexpensive material, that may become the basis of great future industries. In the writer's opinion, carbo-hydrate nations are destined to exercise a controlling role in human affairs through chemical synthesis and fermentation processes.

This paper treats of two such processes, and will be divided in two parts; the first dealing with the production of Acetone and Butanol from waste molasses, and the second with that of normal butyric acid from the same source. While the first part embodies the account of a finished piece of industrial research (in so far as laboratory work is concerned) that was started during July, 1931; the second presents the progress made to date in a new investigation initiated only a few months ago. This fact will account for the lack of definite data in the subject matter of part two.

PART I

BUTANOL AND ACETONE FROM WASTE MOLASSES

The Necessity of Industrial Research in the Industry of the Sugar Cane.—While there exists no single great industry more needful of industrial research than that of the sugar cane, there is none showing less interest and progress in this respect. There have existed several reasons for this attitude, a discussion of which lies beyond the scope

of this article. But whatever reasons existed in the past for this apathetic attitude towards industrial research, they are no longer tenable under the existing general conditions of this great industry. Millions are being wasted every year in the form of unutilized by-products and waste materials while the price of the main product drops to unprofitable levels. It seems that those responsible for the wastes are so close to them that they have come to regard them as unavoidable accompaniment of the industry. Meanwhile the dextrose industry has become a serious competitor, and the commercial production of Levulose promises a still stronger future rival. At the same time the technical world hears of sugar from wood waste in Sweden and Germany, and sugar again, from potatoes in Ireland. Is the cane-sugar industry to remain in the worn-out traditional channels, blind and oblivious to the "New Era" that will revolutionize the world through carbohydrate chemistry?

Possibility of Producing Butanol and Acetone from Waste Molasses.—With a determined purpose of doing his share (no matter how small and inadequate) for the amelioration of this existing trend of affairs, and with a deep rooted conviction that if the sugar industry is going to servive its present crisis, new products must be manufactured either from sugar itself, or its by-products, the writer started his investigation on the problem of the fermentation of final molasses for the production of butanol and acetone. From his search of the literature the writer knew of no existing fermentation process of commercial magnitude for the production of these solvents from Waste Molasses, at the time of the commencement of his investigation, July 1931.

Searching for the Fermenting Organism.—Having learned from the literature on this subject that the organisms responsible for the acetone-butylic fermentation of carbohydrate material were widely distributed in nature, the writer decided to try as possible sources such things as soil, decaying vegetable matter, potatoes, sweet potatoes, beets, and sugar cane. For the culture medium, a dilute solution of final molasses in water was selected, with such chemical and physical modifications as would best suit the growing and development of the given organism and its power for the production of solvents. The word "Solvents" is used throughout this work to include all the valuable liquid products of fermentation.

The molasses solution was placed in test tubes of about 50 ml. capacity, and inoculated in each case directly with small pieces of the material suspected of containing the butyl bacillus. After incuba-

tion for a period of from 36 to 48 hours, the presence of butanol was to be detected first hand, by that most chemical of human senses, the sense of smell. If the characteristic butanol scent could be detected, then further work was to be done to isolate the organism and set it to work under conditions favorable to its growth, development and solvents producing power.

The preliminary work as described above was conducted without success in the great majority of cases; the potatoes, sweet potatoes, and the soils in which they grew giving negative results. The writer reached the conclusion that either these materials did not contain the butyl bacillus, or else, the strains contained in any or all of them could not be grown and developed in the culture medium selected for this work.

The search for the organism was then started among the sugar cane varieties grown in Puerto Rico; and this time luck came to help out the writer's labors. Not less than 50 different varieties and the soil around their roots were examined, and in a little over 20 per cent of these, the organism was found. However, not all of them showed the same characteristics, ability to grow, develop and produce the required solvents in the chosen medium. The apparently most promising strains were obtained from the following canes:—POJ–2725; 2883; 2873; 979; 1228; FC–588; 916; 998; and PR–820 and 807. But the only one that convinced the writer of possessing very remarkable power for doing the work, was a strain obtained from the roots of a cane of the Kassoer variety.

Having thus obtained promising material, the writer proceeded to the work of purification and isolation of the bacillus. This work will be described in the next paragraph.

Isolation of the Bacillus in Pure Culture.—The bacillus found on the roots of the Kassoer cane was isolated as follows: A set of 12 test tubes each containing 10 ml. of sterile mash of 4 deg. Brix density, were inoculated with small amounts of the unsterilized cane roots. The tubes were then immersed in boiling water for 50 seconds and immediately cooled in running water. They were then incubated for 36 hours and examined for odor and gas production. The tube showing most vigorous fermentation and strongest butyl odor was chosen, and further incubated until fermentation was completed; reheated in boiling water, cooled, and the contents plated in malt gelatine agar. After inoculation of the malt gelatine agar, the medium was poured into the inverted lid of a sterile Patri dish, and the bottom section of the dish was then floated on the liquid. After incubation the two

parts of the plate were easily separated and the colonies fished. These colonies were used for further propagation as found necessary.

Adapting the Medium to the Bacillus.—Lack of inverting power and inability to attack sucrose for the production of solvents were soon discovered in the bacillus. The bacillus, on the other hand, readily attacked and decomposed reducing sugars with the production of the desired solvents. Hence the sucrose in the molasses used for making up mashes was always inverted before inoculation.

After some experimental work details of which would occupy too much space, the conclusion was reached to make the mashes according to the procedure described below, calling this mash "The Standard Mash".

Ninety grams final molasses were weighed into a two liter Erlenmeyer flask, four hundred ml. distilled water added and the molasses dissolved by heating on a water bath with occasional shaking. When solution was effected, 1.5 ml. strong sulphuric acid were added, and the flask was autoclaved during half hour at 20 pounds pressure to effect inversion. After cooling to about 50 deg. C., five and a half grams of calcium carbonate, and 1.5 ml. strong ammonia water were added to the mash in the flask. When effervescence subsided, the mash was completed to 1,800 ml. with distilled water, plugged with non-absorbent cotton, and again autoclaved for half hour at 20 pounds pressure. When thus prepared, this standard mash gave a reading of 90 mv. at the potentiometer, equivalent to pH–6. Any small variation from a potentiometer reading of 90 mv. was corrected by addition of hundredth normal sulphuric acid or sodium hydroxide; as found necessary. The bacillus worked very satisfactorily in this medium.

Fermentation Tests.—Many fermentation tests using the pure culture obtained and the standard mash prepared as described above were conducted at various temperatures, ranging from 30 to 40 deg. C. to ascertain the optimum temperature of fermentation. Results of those tests showed that the optimum temperature for a successful fermentation was 35 deg. C. At this temperature the greatest yield of solvents was obtained, fermentation being completed in from 48 to 54 hours after its initiation. When working at temperatures above 35 deg., C. it was found that fermentation was rapid and vigorous; but the yield of total solvents was less than when working at 35 deg. C., while the ratio of acetone to butanol was higher. At 39 deg. C. the organism worked very briskly for some time but all action soon ceased, and when examined under the microscope the bacilli had ap-

parently lost their characteristic motility, very few vegetative cells being observed. When temperatures from 30 to 35 deg. C. were used the fermentation was rather sluggish, and instead of from 48 to 54 hours, it took from 60 to 72 hours to complete it. The table on next page shows the influence of temperature during the fermenting period.

TABLE SHOWING THE EFFECT OF TEMPERATURE DURING FERMENTATION

% T. S. = PERCENT TOTAL SOLVENTS

Test No.	Temperature of Fermentation Deg. C.	Time of Active Fermentation Hours	Analysis of Products				Ratio of Butanol to Acetone	
			T. S. %	Butanol %	Acetone %	Ethanol %		
1.....	30	70	23.50	17.82	4.95	0.73	3.6	I
2.....	31	66	24.05	18.00	5.00	1.05	3.6	I
3.....	32	63	24.50	18.28	5.07	1.15	3.6	I
4.....	33	60	25.80	19.31	5.51	0.98	3.5	I
5.....	34	57	27.10	20.27	5.78	1.05	3.5	I
6.....	35	49	29.50	21.98	6.27	1.27	3.5	I
7.....	36	45	27.65	20.07	6.08	1.50	3.3	I
8.....	37	38	24.49	17.54	5.68	1.28	3.1	I
9.....	38	32	18.90	12.98	4.99	0 93	2.6	I
10	39	25	12.00	7.57	3.78	0.65	2.0	I

The above figures show that the optimum temperature for fermentation is 35 deg. C.; that below this temperature fair results may be obtained down to about 32 deg. C. and up to about 37 deg. C. Temperatures above 37 deg. C. give very poor results as to total yields and butyl alcohol-acetone ratio; while temperatures below 32 deg. C. though not giving very bad yields, take too long to finish fermentation.

Acidity of Mashes.—The acidity of all mashes was controlled by pH determinations using the potentiometer and quinhydrone electrode. Having found that the bacillus worked best under slightly acid conditions, the initial pH of all mashes was regulated to a reading of 90 mv. as stated previously when describing the "STANDARD MASH".

An experiment was run to determined the variations in acidity during and upon completion of fermentation.

During a normal fermentation at optimum temperature, it was observed that the mv. reading rose steadily as soon as fermentation started, continuing this rise during the first 22 or 24 hours, when a potentiometer reading of about 160 mv. could be observed. After this concentration of acidity was reached, the mv. readings started to yield descending values till a constant or nearly constant value of from 125 to 130 mv. was obtained which remained so until fermentation stopped.

In cases of a poor fermentation test due to contamination of the inoculum, or of the mash after inoculation, or to any other inhibiting force, the acidity continued to rise going up to readings between 190 and 210 mv. Every time that this happened, poor yields of solvents were to be expected; examination of a sample under the microscope showing less motility of the organism, signs of contamination; and in extreme cases the organism disappeared altogether from the field of vision.

Thus the determination of pH values periodically (say every 2 or 3 hours) proved to be an excellent control, as an abrupt rise in mv. readings was a sure indication of forecoming trouble. The following curves show the rise in pH during a (I) normal, vs. a poor (2) fermentation.

The following table offers some figures obtained from fermentation test conducted at optimum temperature using "Standard Mash".

DATA

Hour and date of inoculation ..... 10 A. M. Oct. 1, 1931
Hours after inoculation at which fermentation started ..... 4 hours
Date of completion of fermentation ..... 3 P. M. Oct. 3, 1931
Hours of active fermentation 53
Incubating temperature ..... 35 deg. C.

Mash No.	Starting Bx.	Final Bx.	Starting Mv.	Final Mv.	S. G. of Distillate at 20/4 C.	% T. S.	% Butanol	% Acetone
1.....	4.60	2.7	95	152	0.9940	20.55	15.88	4.55
2.....	4.70	2.7	93	155	0.9937	22.27	16.65	4.60
3.....	4.60	2.3	88	130	0.9923	30.70	22.64	6.40
4.....	4.60	2.5	90	153	0.9940	20.55	16.00	4.40
5.....	4.60	2.2	89	125	0.9922	31.36	22.76	6.60
6.....	4.70	2.4	87	132	0.9930	26.33	19.22	5.89
7.....	4.65	2.6	93	142	0.9935	23.20	17.59	4.35
8.....	4.60	2.6	89	151	0.9937	22.27	17.24	3.98
9.....	4.60	2.6	85	145	0.9938	21.82	16.42	3.87
10.....	4.60	2.4	86	135	0.9933	24.44	18.19	4.92

NOTE: — The percentage ethanol produced may be found by subtracting from % T. S. the sum of the percentages of butanol & acetone.

Determination of Optimum Sugar Concentration.—From an industrial point of view it was thought very important to determine the optimum sugar concentration in the mash at which the organism would work efficiently.

A series of 12 mashes varying in sugar concentration from 3.65 g. of total sugar to 11.25 grams per 100 ml. of mash were inoculated with the organism, using the same seed for all. After completion of the incubation period, the following results were obtained:

Mash No.	Concentration of T. Sugars Grams per 100 ml.	No. of hours to complete Fermentation	Temp. of Ferm. 35 deg. C.	% Total Solvents on wt. of sugars	% Acetone on wt. of sugars
1	3.65	48	"	24.75	5.21
2	4.25	48	"	27.74	6.09
3	4 55	50	"	27.30	6.12
4	4.85	50	"	27.12	6.22
5	5.14	52	"	22.85	5.20
6	5.45	60	"	10.10	2.50
7	5.75	66	"	7.39	2.00
8	6.05	72	"	4.13	1.15
9	7.25	80	"	1.72	0.65
10	8.50	No ferment			
11	9.75	" "			
12	11.25	" "			

From a study of the data in the above table, it is apparent that best results are obained when working with sugar concentrations of from 4.25 to 4.85 grams per 100 ml. of mash. Fair results may be obtained with concentrations of 3.65 and 5.14 grams of sugar per 100 ml. of mash; but the commercial optimum would be attained when working with a concentration of 4.85 grams of sugar per 100 ml. of mash. With this concentration every 100 ml. of mash will yield on distilling 1.315 gram of total solvents or about 1.65 c. c. It may be observed that mashes Nos. 2 and 5 give slightly higher yields per sugar unit; but having lower concentrations, the actual weights of solvents obtained per 100 ml. of mash are smaller. Also the plant capacity for fermentation and distillation would have to be larger if these concentrations were used commercially. The yields in grams and ml. of solvents per 100 ml. of mash in the cases of the best three concentrations as represented by mashes Nos. 2, 3, and 4 follows:

Mash No.	Grams T. S. per 100 ml of mash	Ml. total solvents per 100 ml. of mash
2	1.18	1.48
3	1.24	1.55
4	1.315	1.65

Activating Agents.—Another fermentation test was run to find the effect of Lamp-Black and Kieselguhr when used in the preparation of the mash separately, and together, in varying proportions. The results obtained are shown in the table below:

TOTAL WEIGHT OF ALL MASHES: 1000 GRAMS

Mash No.	% T. Solids on Wt. of Mash	% T. Sugars on Wt. of Mash	Lamp-Black used Grams	Kieselguhr used Grams	Total Solvents	% Butanol
1	8.8	4.85			24.60	19.07
2	"	"	1		26.50	21.19
3	"	"	2		28.50	23.05
4	"	"	3		25.10	19.41
5	"	"	4		24.60	18.68
6	"	"	5		22.00	16.17
7	"	"		1	27.70	21.68
8	"	"		2	27.70	21 85
9	"	"		3	25.10	19.10
10	"	"		4	25.10	18.95
11	"	"		5	25.10	18.87
12	"	"	1	1	27.80	23.60
13	"	"	2	2	30.00	25.41
14	"	"	3	3	28.70	23.01
15	"	"	4	4	30.00	24.40
16	"	"	5	5	30.00	24.25

A perusal of the table on page 15 will reveal at a glance the beneficial effect obtained by the use of the activating agents discussed on that page. The effect is more striking when using both Lamp-Black and Kieselguhr together. In case when Lamp-Black is used alone, the maximum increase in yield of solvents is obtained by using 2 grams of this substance; and this increase amounts to 3.9 per cent on the weight of sugars in the mash or 15.86 per cent over the yield obtained when no activating agent is used. Using quantities much above this maximum give very little increase, no increase, or even deleterious results; for instance, when 5 grams lamp-black were used the yield of solvents was actually less than when using no activating agent at all. In the case of Kieselguhr alone the maximum increase was obtained when using either 1 or 2 grams of the substance; no further increase being obtained by using larger amounts. In this case the increase in yield amounts to 3.10 per cent on the weight of sugars, or 12.6 per cent over the yield obtained without using activating agents. A maximum yield is obtained when using both activating substances together in the proportion of two grams of each; and in this case the increase amounts to 5.40 per cent on the weight of sugars, or 21.81 per cent over results obtained when using no activating agents.

These results show that in a commercial process it would pay to use these activating substances, as the increase in yields are remarkable, especially when using both activating substances together.

Industrial Possibilities.—The writer's opinion is that the Bacillus ACETOBUTYLICUM discovered and isolated by him could have profitable industrial application; and this opinion is based on the following facts:

1. So far there exists no large scale production of these solvents using FINAL SUGAR HOUSE MOLASSES as the raw material.

2. Final molasses is to-day the cheapest carbohydrate source in the market, its price in Puerto Rico being about 3.5 cents a gallon, delivered at the various mills. A gallon of Puerto Rican final molasses contains an average of six pounds total sugars.

3. This bacillus is able to produce more yield of solvents per unit of sugar content of the fermenting medium than any other known so far.

4. The bacillus which is most widely used commercially to-day is that of WIEZMANN, and it produces butyl alcohol, acetone, and ethyl alcohol in the ratio 6:3:1, respectively using corn mash. This bacillus has not been able to produce these solvents profitable from mo-

lasses mash. The writer's bacillus produces these same solvents in the ratio 14:4:1, respectively, using final molasses mash of from 8 to 9 deg. Brix, corresponding to a sugar concentration of from 4.5 to 5.0 per cent.

5. The maximum actual laboratory yield obtained from 1 gallon FINAL MOLASSES by the writer has been:

Total solvents	30.00 per cent;	or 1.95 pounds
Butyl alcohol	22.31 per cent;	or 1.45 pounds
Acetone	6.15 per cent;	or 0.40 pounds
Ethyl alcohol	1.54 per cent;	or 0.10 pounds

The present average market value of the above products, per pound, are 10, 9, and 4 cents, respectively, for butyl alcohol, acetone and ethyl alcohol. There are good reasons to believe that these prices are at about the lowest possible level and that there exists great probability of a future rise in all of them. Even at these low prices, a gallon of sugar factory final molasses would yield a gross value of 18.5 cents when used in the production of these solvents. The actual total cost of production should not exceed 10 cents per gallon of molasses worked up, including all expenses. These expenses may be lowered considerably by working the process as an adjunct to a sugar factory, where cheap fuel in the form of bagasse would be available and where no transportation charges would have to be paid on the molasses used.

Patent applied for by author.

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PART II

NORMAL BUTYRIC ACID FROM WASTE MOLASSES

Butyric Acid is a Valuable and Important Acid.—Butyric acid is one of the most valuable aliphatic acids used commercially. Its steady price offers a striking contrast with the declining values that have been prevalent in the market for chemical products during the last four or five years. The present carload price in the United States for this commodity is 80 cents per pound on the basis of 100 per cent acid.

This attractive price led us to investigate the possibility of producing this organic acid from cane factory waste molasses by microbiological methods. The success obtained in our previous investigation on the production of butanol and acetone by similar methods, encouraged us to try this new study.

Difficulties Encountered by Previous Investigators.—Although fermentation processes leading to the production of organic acids have been practiced before the beginning of civilization, the mechanism whereby these processes took place on the agents responsible for them were utterly ignored. The production of butyric acid, the agent responsible for rancid butter, remained in the situation common to all these processes until Pasteur recognized the butyric fermentation as a well defined microbiological phenomenon; describing this fermentation as an anaerobic process before the Academy of Science in Paris, 1861.

Since this date, many groups of these organisms have been found. and their products of fermentation studied by several well-known investigators, among which we may mention Kirov; Baier; Fitz; Winogradsky; Buchner and Meisemheimer. These men of science agreed in that the formation of butyric acid by fermentation when using the organisms known to them, was generally accompanied by secondary reactions, producers of a variety of other substances. For instance, Buchner and Meisenheimer, when working with the "Bacillus Butyricus" Fitz, found the following products as typical of the fermentation of 100 grams glucose:—0.7 grams butanol; 2.8 grams ethanol; 1.6 grams hydrogen; 3.4 grams formic acid; 10 grams lactic acid; 7.5 grams acetic acid; and 26 grams butyric acid.

Recently, the chemists H. T. Herrick and O. E. May of the Department of Agriculture, Washington, D. C., published a circular on the production of organic acids by fermentation in which they opined that the butyric fermentation has not been applied industrially in commercial magnitude due to the great variety of substances, other than butyric acid produced during the fermentation.

The patent literature describes some processes which give the impression that the work has been done in decidedly empirical form. In some cases it is really difficult to understand how a patent could be secured on such vague, indefinite and entirely unscientific data.

Attacking the Problem.—Having acquired from the literature a knowledge of the butyric fermentation, whose synopsis is given above, we resolved to attack the problem of butyric acid production from waste or "final" sugar factory molasses, using a native bacillus.

The Bacillus is Found.—At the time of our determination to work on the butyric fermentation, we were preparing a series of extracts from the Annatto Seed (Bixa Orellana) that were to be sent to the Chicago Fair. A few seeds left over the week end in a test tube with distilled water to which a little sodium carbonate had been added, were found on next Monday morning in a very active state of fermentation. On further examination it was found that the fermenting liquid had turned from an alkaline to a decided acid reaction. Moreover, a strong butyric acid scent could be noticed. We had found the organism needed for our intended work on waste-molasses.

Our Preliminary Work.—We had learned from the experience of previous workers in this field, that the problem of the commercial production of butyric acid by microbiological methods would be practically solved with the discovery of a fermenting organism capable of producing the desired fermentation free of the secondary products obtained when working with organisms already known.

Hence, our greatest interest, once the bacillus was found, was to find out whether or not the organism would satisfy the requirements as to yield and purity of the main product of fermentation. So, as soon as we had enough quantity of the product, an analysis was made to determine its degrees of purity. The results were so highly satisfactory that a duplicate sample was sent to the Bureau of Chemistry of the Department of Agriculture, Washington, D. C. The sample was sent in the form of the barium salt of the acid. The report received from that Department stated that the product was practically entirely butyrate of barium, and that the free acid showed a high degree of purity.

These results were, of course, sufficient to give a great technical and commercial interest to our bacillus; for we had learned that the inhibiting factor militating against their commercial application, was the heterogenous product of fermentation found when working with other butyric ferments.

Other Pertinent Facts Found to Date.—As stated in our introduction this investigation is merely starting, so no definite data is available as yet. But from what has been done to date, the following additional facts may be stated:

1. The organism is exceptionally vigorous, and has shown itself capable of competing with other organisms that may gain access to the fermenting liquid. This may indicate that an absolutely pure culture is not indispensable for the growth, development, and acid production of the organism. From a commercial standpoint this is

an important factor favoring the organism; for the difficulties encountered in plant work in the prevention of contaminations in pure cultures are only too well known.

2. The organism is facultative anaerobic. This is another point of great technical importance, for strict anaerobes bring great difficulties of a technical nature, besides complications of equipment when an effort is made towards their commercial exploitation.

3. We have not determined as yet the optimum conditions of fermentation, nor the maximum obtainable yields of acid; but in the preliminary tests effected up to this time, the yields of acid have varied between 30 and 40 per cent in round numbers. These yields have been calculated on the weight of total sugars contained in the fermenting mass.

4. Accepting an average value between the two figures given above, as the probable commercially obtainable yield, we would have that a gallon of "final" or waste molasses, would yield about 2.1 pounds of the acid with a gross value of $1.68.

Finale.—As closing words I wish to express my firm conviction in the glorious future awaiting fermentation processes in the field of modern industrial chemistry, and especially in their application to carbohydrate materials. Indeed, many of our more important organic chemical products will be manufactured in a not remote future by methods of industrial microbiology.

The beautiful islands of the Caribbean offer unexplored treasures in their microbiological flora, and are destined to become great chemical laboratories of the future where fermentation industries will be predominant; and the future of our great sugar industry will be most intimately connected with such methods of manufacture.

Patent will be applied for by the author.

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